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(54) Title: PRIMARY BILIARY CIRRHOSIS AUTOANTIGEN

(57) Abstract

A synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of primary biliary cirrhosis, or an antigenically active fragment thereof, is produced by recombinant DNA techniques. The synthetic peptide or polypeptide, or fragment, may be used in the treatment of patients or in diagnostic tests, particularly for the detection of anti-mitochondrial antibody in a serum sample.

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PRIMARY BILIARY CIRRHOSIS AUTOANTIGEN

This invention relates to the identification, cloning and expression of an auto-antigen which is recognised as a target in the characteristic autoantibody response in primary biliary cirrhosis (PBC), and to the use of this protein, fragments thereof or fused polypeptides containing the protein or fragments thereof in diagnostic tests for PBC, and in treatment of patients suffering from PBC.

Primary biliary cirrhosis (PBC) is a chronic disease characterised by progressive inflammatory obliteration of the intrahepatic bile ducts. The disease is marked by an autoantibody response to mitochondria [1-4], originally identified using immunofluorescence[5]. With the recent use of immunoblotting, specific proteins have been

recognized as targets of the anti-mitochondrial antibodies (AMA) of PBC[2,6,7]. In particular, serum antibodies to a 70 kilodalton (kd) protein have been found in greater than 95% of patients with PBC but not in patients with other autoimmune liver diseases,[2,8]; two other proteins of 45 and 39kd are less frequently detected in PBC sera[1,2,9]. The identity of each of these autoantigens has been unknown, as is the relationship of these antigens to the pathogenesis of the disease. However, the 70kd antigen is highly conserved in evolution, being present in mammals, yeast and bacteria[10] and it is therefore believed to have an important structural or biological function[2].

Despite the paucity of data on mechanisms of anti-mitochondrial antibody formation, by enzyme-linked immunosorbent assay (ELISA), clinically more than 95% of patients with PBC can be found to have such anti-mitochondrial antibodies [2,6]. When crude mitochondrial antigen preparations are used, subjects with a variety of diseases, including patients with liver diseases other than PBC, certain connective tissue diseases, and drug reactions, and occasionally even healthy individuals, can also be demonstrated to have antibodies to mitochondria. Accordingly, assays using such crude preparations are unable to provide specific diagnosis of PBC. By way of example, German Patent Publication No. 3,237,602 discloses an ELISA for detection and determination of antimitochondrial antibodies in serum using a crude mitochondrial antigen preparation. The lack of specificity of the assay is evident from the suggested use of the assay in the specific diagnosis of disorders such as PBC as well as the cholestatic form of chronic active hepatitis, syphilis (II), drug-induced pseudo lupus erythematodes syndrome, certain primary non-hepatic

immunopathies, iproniazid-induced hepatis and side effects of certain medicaments such as beta-receptor blockers. By more vigorous isolation of mitochondrial membranes, the problem of antigenic heterogeneity becomes clearer and has led to definitions of specific mitochondrial antigens based on trypsin sensitivity and location of antigens within inner vs outer mitochondrial membranes. Notwithstanding this, however, the diagnosis of PBC still relies heavily on the demonstration of anti-mitochondrial antibodies by the relatively insensitive procedure of immunofluorescence or by more sensitive, but still relatively nonspecific, methods, including complement fixation, ELISA, and immunoprecipitation [23-28].

The present invention is based on the identification of a cDNA clone derived from a rat liver gene expression library that expresses the 70kd mitochondrial autoantigen of PBC, (called M2 by some groups of investigators[1,9]) and on sequence determination thereof. The sequence is encoded by nuclear and not mitochondrial DNA.

The present invention thus provides the basis of an extremely sensitive and specific diagnostic ELISA for anti-70kd antibodies found in PBC.

According to a first aspect of the present invention, there is provided a DNA molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the base sequence coding for the 70kd mitochondrial autoantigen of primary biliary cirrhosis (PBC), or an antigenically active fragment thereof.

Preferably, the DNA molecule in accordance with this aspect of the invention is characterised by at least a portion thereof comprising a base sequence substantially as shown in Figure 6, or a fragment thereof.

In another aspect, this invention provides a

recombinant DNA molecule comprising a nucleotide sequence as described above, operatively linked to an expression control sequence. Such a recombinant DNA molecule may for example comprise an expression vector such as a bacteriophage or plasmid, or a host cell such as a bacterium or other microorganism transformed therewith.

In yet another aspect of this invention there is provided a synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of primary biliary cirrhosis, or an antigenically active fragment thereof.

Preferably, the synthetic peptide or polypeptide of this aspect of the invention is characterised by at least a portion thereof comprising an amino acid sequence substantially as shown in Figure 6 or Figure 8, or an antigenically active fragment thereof.

Such a synthetic peptide or polypeptide may, for example, be prepared by expression of a host cell transformed with a recombinant DNA molecule as broadly described above, either as a fused polypeptide or directly. Alternatively, it may be prepared by chemical synthesis, such as by the well-known Merrifield solid-phase synthesis procedure.

The present invention extends to the synthetic peptide corresponding to the entire 70kd autoantigen, and to nucleotide sequences coding for the entire autoantigen, as well as to fragments thereof. By way of example, one such fragment is the fragment encoded by nucleotides 76-679 of Figure 6. This fragment of approximately 200 residues is capable of adsorbing out of a patient serum all antibodies directed against the native autoantigen. Within this fragment is a 20 residue fragment of amino acid sequence:

A E I E T D K A T I G F E V Q E E G Y L

which has been demonstrated to have substantial reactivity with autoantibodies. This fragment is common to the sequences of both Figure 6 and Figure 8. The present invention therefore extends to the use of antigenically active fragments such as these, as well as to the use of the entire autoantigen, in diagnostic assays.

The present invention also extends to the use of the synthetic peptide or polypeptide, or fragment, of this invention as an antigen in a diagnostic test for PBC by detection or determination of the titre of antimitochondrial antibody in a patient's serum, for example using ELISA or RIA technology or an agglutination assay using antigen-coated beads or the like. The invention also extends to use of the synthetic peptide or polypeptide, or fragment, in the treatment of patients. In this latter aspect, such methods of treatment include the use of the synthetic antigen, as an adsorbent to remove PBC antibodies or reactive cells from a patient, as well as the use of these active components in direct administration to a patient as a desensitizing agent to eliminate or diminish reactivity of the patient to the PBC autoantigen.

In addition to use of the synthetic autoantigen in the detection of anti-mitochondrial antibody in a serum sample, the present invention extends to use of the synthetic peptide or polypeptide, or fragment, in the measurement of class-specific immunoglobulin titres using specific typing reagents. Applications also extend to the measurement of the affinity of either the whole autoantibody, or the affinity of individual classes or subclasses of the autoantibody. Affinity may be measured by a number of procedures, for example, by replicate ELISA assays performed using different washes of guanidine thiocyanate[42]. A further extension of the diagnostic

assay is the measurement of the degree of interference of autoantibodies with the enzymic function of the 70kd autoantigen (now shown to be lipoate acetyl transferase, see later). The source of the enzyme may be derived from expression of full length clones as native polypeptides or fusion polypeptides, or from expression of enzymatically active fragments or purified protein from mitochondria. The enzyme assay is a standard assay well known in the art, but modified to include a step of incubation with sample serum or cells. In yet a further extension of the use of the synthetic peptide or polypeptide, or fragment, there is included the measurement of reactivity of patient cells to the autoantigen. The synthetic peptide or polypeptide, or fragment, may be added, in solution or bound to a solid support, to patient cells derived from peripheral blood or from tissue biopsies either unfractionated, fractionated or as a continuous cell line. Reactivity to the autoantigen may then be measured by standard proliferation assays such as incorporation of tritiated thymidine, standard cytotoxic assays such as release of marker radioactivity from target cells, or other standard assays of cellular reactivity which are well known in the art.

In one particularly important aspect of this invention, there is provided a diagnostic test for detection of antimitochondrial antibody in a serum sample, which comprises the steps of:

(i) contacting said serum sample with a synthetic peptide or polypeptide displaying portion of the 70kd mitochondrial autoantigen of PBC, or an antigenically active fragment thereof, said synthetic peptide or polypeptide being immobilized on a support, and

(ii) detecting the presence of anti-mitochondrial

antibody in said serum bound to said synthetic peptide or polypeptide.

In this aspect, the invention also provides a diagnostic test kit for detection of anti-mitochondrial antibody in a serum sample, which comprises:

(i) a support having immobilised thereon a synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of PBC, or an antigenically active fragment thereof, and

(ii) means for detecting the presence of anti-mitochondrial antibody in said serum bound to said synthetic peptide or polypeptide.

Preferably the detection of the presence of bound AMA is by use of well known RIA or ELISA techniques.

As a result of the production of a recombinant fused polypeptide displaying the antigenicity of the 70kd mitochondrial autoantigen of PBC, this autoantigen has now been identified as lipoate acyltransferase. In addition, the immunoglobulin isotypes of the anti-mitochondrial antibodies has been determined, and IgG3 has been found to be the predominant isotope in a group of PBC patients, with IgM next most prevalent. Comparison of serum immunoglobulin isotype levels of PBC patients with healthy normal adults has shown that serum IgG3 and IgM were very elevated in PBC; IgG3 at 5.5 fold and IgM at 4.3 fold above normal.

In accordance with the present invention, expression of the cDNA insert encoding the mitochondrial autoantigen, or fragments thereof, may be achieved in a number of different ways. The detailed description herein provides examples of expression as β-galactosidase fusion proteins in the vectors λgt11 and pBTA224, using as host cells E.coli strains such as JM101, JPA101 and

7118. Successful expression of the autoantigen as a fusion protein may also be achieved using the well-known PVC vectors, or using the pGEX series which give expression of glutathione S-transferase fusion proteins, again using E.coli as the host cells. Alternatively, the mitochondrial autoantigen may be expressed as a non-fused polypeptide, by using appropriate vector and host cell combinations. Other vector and host cell combinations which can be used in accordance with the present invention including a number of well described yeast shuttle vectors growing in yeast cells, or eukaryotic vectors in continuous cell lines, or transgenic animals.

The identification, cloning and expression of the 70kd mitochondrial autoantigen of PBC in accordance with the present invention, and its use in an ELISA, will now be described in detail, with reference to the accompanying drawings in which:

Figure 1 shows specificity of the fused polypeptide. In lanes A and B, two different PBC sera at a dilution of 1/1000 were probed against lysates of pRMIT transformed JM101 cells. Both sera reacted with a polypeptide at 160kd. In contrast, in lanes C and D, the same sera were nonreactive when probed against lysates of control cells containing an irrelevant insert that is also fused to β-galactosidase. The reactive bands in lanes C and D correspond to E.coli proteins. Duplicate blots probed with normal sera at 1/100 and 1/1000 failed to detect the fused polypeptide and are not shown. There is some breakdown of the fusion protein with reactivity at 36kd.

Figure 2 shows identification of the pRMIT fused polypeptide. The reactivity of absorbed and unabsorbed PBC serum against human placental mitochondrial proteins

after PAGE was determined. In lane A, the probe was an unabsorbed PBC serum at a final dilution of 1/2000. In lane B, the probe was the same serum at a final dilution of 1/2000 after extensive absorption for 72hr against cells transformed with non-recombinant pBTA224 and passage over a solid support to which had been bound a lysate of cells transformed with non-recombinant pBTA224. In lane C, the probe was the same serum at a final dilution of 1/2000 after absorption for 72hr against cells transformed with non-recombinant pBTA224 and passage over a solid support to which had been bound a lysate of cells transformed with expressing pRMIT. The serum was also studied at 1/200 and 1/20,000 (Table II).

Figure 3 shows specificity of affinity-purified antibody. In lane A, an unabsorbed PBC serum at 1,2000 was probed against placental mitochondria, reacting with both the 70 and the 45kd protein. In lane B, the column eluate was probed against the same mitochondrial preparation. Note the reactivity was only to the 70kd protein, and the reduction in signal correspond to the expected recovery for such elution. Even on a very long autoradiographic exposure time of 1wk, there remained activity only to the 70kd protein (data not shown). In lane C, the eluate was probed against a sonicate of induced JM101 transformed with pRMIT. The intensity of the 160kd fused polypeptide was due to the large quantity of fused polypeptide expressed. In lane D, the eluate was probed against a sonicate of induced JM101 transformed with an irrelevant plasmid that encodes an abundant fused polypeptide.

Figure 4 shows immune response of BALB/c mice immunized with pRMIT induced fused polypeptide. Placental mitochondria were separated by PAGE on a 7.5% gel and blotted onto nitrocellulose, and the fractionated proteins

were probed with sera at a dilution of 1/1000 (lane A) or with serum from a patient with PBS at 1/1000: immunized mice produced antibody against the 70kd but not the 45kd protein.

Figure 5 shows immunofluorescence of HEp-2 cells. BALB/c mice were immunized with the purified fused polypeptide and sera incubated with HEp-2 cells. Note the typical mitochondrial pattern of reactivity.

Figure 6 shows nucleotide sequence of pRMIT and deduced amino acid sequence of the 70kd mitochondrial antigen of PBC.

Figure 7 shows comparison of sensitivity between the ELISA (+) and immunofluorescence (□) in detection of AMA in PBC. PBC sera were tested at every 10 fold dilution stating from 1:1000 in the ELISA whereas in the immunofluorescence against Hep-2 cells every 2 fold dilution starting from 1:10 was used. The positives on ELISA were defined as 2 S.D. O.D. units above the mean for normal sera.

Figure 8 shows the nucleotide sequence and deduced amino acid sequence of a 2.2kb cDNA insert that encodes the human equivalent of the sequence depicted in Figure 6, encompassing the human equivalent of the region of nucleotides 105-1065 in Figure 6. This human cDNA clone was obtained by probing a human placental library using pRMIT as a hybridization probe according to known techniques. The sequences are highly homologous and have comparable reactivity with auto-mitochondrial antibodies; accordingly either antigen sequence could be used as the basis of a diagnostic test to detect anti-mitochondrial antibodies or auto-reactive cells.

A. MATERIALS AND METHODS.

Screening cDNA library.

A rat liver cDNA library in λgt 11-Amp3 consisting of 15,000 recombinants, of average length of 1.4kb, was probed by using sera from patients with PBC. The sera used for screening were from each of three patients with classical PBC who were shown to have antibodies to mitochondria by immunoblot analysis of electrophoretically separated proteins of human placental mitochondria[2]. As some patients with PBC have high-titre antibodies to E.coli, the sera were extensively preabsorbed against E.coli infected with non-recombinant phage. The sera were used for probing at a final concentration of 1:1000[11,12]. The λ-Amp3 library was incubated with E.coli, strain ST9, for 15min at 37°C and then was plated for 2hr at 42°C in LB agar. Thereafter, nitrocellulose filters that had been previously soaked in 10ml isopropyl-thiogalactosidase (IPTG) and allowed to air dry were overlayed on each plate. The plates were then incubated overnight at 37°C. The nitrocellulose was removed after alignment and was washed for 1hr in PBS with 5% milk powder, pH 7.4. The filters were then incubated for 45min with previously absorbed sera of patients with PBC, washed three times for 30min, and incubated with ^{125}I-protein A (300,000 cpm/ml) for 45min. Finally, the filters were washed three times, were allowed to air dry, and were placed on XRP-1 film with an intensifying screen for an overnight (12hr) exposure. All washings and dilutions of sera and ^{125}I-protein A were done with milk powder. Putative positive clones were picked and rescreened for plaque purification[12,13].

Subcloning.

Three clones gave positive signals, a frequency of approximately one in 50,000 clones. These positive clones were plaque purified. Each of these clones yielded an identical sized insert of approximately 1.4kd. The

inserts were subcloned in the plasmid vector pBTA224, which is a high copy plasmid expression vector with a site for insertion of foreign DNA identical to that of λ-Amp3. Therefore, 50% of the subclones should also give a positive signal on an immunoassay as the insert is in the same reading frame as λ-Amp3. Clones expressing an unrelated rat liver cDNA (the F alloantigen) were used as controls. Arrays of pBTA224 colonies were prepared to identify immunoreactive clones. Colonies were incubated for 16hr at 37°C, then were induced with 10mM IPTG for 4hr. The colonies were lysed and prepared for antibody probing as described[11]. Filters were probed with either a 1/1000 dilution of absorbed PBC sera or a 1/100 dilution of normal serum. One positive clone, designated pRMIT, that expressed a fused polypeptide of 160kd was selected for further study.

Immunoblotting of mitochondrial proteins.

Mitochondria from human placenta were prepared as described[2,14]. Polyacrylamide gel electrophoresis (PAGE) was performed on 1mm-thick slab gels in 0.1% SDS, using a 3.8% stacking gel and a 10% resolving gel. Before PAGE, the purified mitochondria were suspended at a concentration of 4mg protein/ml and were incubated for 30min with 10,000 U of bovine pancreatic DNAse 1 at 37°C, and then were held with an equal volume of 3% aqueous octyl glucoside for 15min at 4°C. The final preparations were diluted with Tris-HCl, pH6.8, containing 4% SDS, 20% glycerol, and 5% 2-mercaptoethanol (sample buffer) and were boiled for 5min. Approximately 10µg protein were loaded in each gel lane[2].

Specificity of pRMIT fused polypeptide.

To demonstrate that pRMIT expressed an antigen specifically reactive with sera from patients with PBC, lysates of the expressing clone were probed with sera from

healthy persons or from patients suffering from different autoimmune diseases. Briefly, a 100ml overnight culture of JM101 cells transformed with pRMIT was diluted 1/10 in L-broth containing 10mM IPTG. Four hours later the cultures were spun at 5000xG for 10min and were snap frozen after addition of 20ml of phosphate-buffered saline. PAGE was performed on 1mm-thick slab gels with 0.1% SDS, using a 3.8% stacking gel and a 7.5% resolving gel. Samples were diluted 1/100 in the above sample buffer and were boiled for 5min. Each lane contained approximately 5 to 10µg of protein. The samples were probed with PBC sera diluted at 1/1000, and the reactivity was determined as above, using ^{125}I-protein A and exposure for 18hr. These same sera also were used to probe immunoblots of lysates of non-recombinant control clones or clones expressing a fused polypeptide coded by an irrelevant DNA insert. The sera used were from patients with PBC, systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, chronic active hepatitis and from healthy normal volunteers. All control sera were studied at a dilution of 1/100.

Identification of fused polypeptide.

The fused polypeptide expressed by pRMIT was characterised to determine whether it was a mitochondrial antigen recognised by PBC sera. The clone pRMIT was grown in liquid culture overnight. It was thence put into log phase and induced to give maximal expression of the fused polypeptide with 10mM IPTG for 4hr. Bacterial lysates were prepared as above and coupled to cyanogen bromide-Sepharose[15]. This solid support was then used as an affinity reagent to bind antibodies selectively from seven different PBC sera. First, sera from seven patients with PBC were absorbed extensively with sonicates of E.coli transformed with non-recombinant pBTA224. Thence,

the sera at dilutions of 1/200, 1/2000, and 1/20,000 were passed through the lysate of pRMIT-transformed bacteria bound to a solid support. The nonabsorbed antibodies were collected, compared with unmanipulated sera at the same final dilution, and used to probe placental mitochondria, prepared as above.

Preparation of affinity-purified antibody.

Affinity-purified antibody was prepared by first extensively preabsorbing five different reactive sera with sonicates of JM101, which had been transformed with non-recombinant pBTA224, and then passing this absorbed serum over a column of JM101 transformed with non-recombinant pBTA224[15]. Each serum was passed over a column of induced JM101 cells transformed with pRMIT, and the column was washed for 24hr with 100-fold the bed volume of the column. Thence, lycine HCl was used to elute the bound antibodies[15]. The antibodies that had bound to the pRMIT absorbent were probed against fractionated placental mitochondria, a lysate of expressing pRMIT, and a lysate of a control recombinant clone. They were also reacted by immunofluorescence with either HEp-2 cells or kidney tissue sections.

Isolation of mitochondrial antigen expressed as fused polypeptide.

Isolation of the fused polypeptide was performed by using gel filtration in the presence of SDS to fractionate the insoluble pellet and to obtain material suitable for immunization. A clone of pRMIT was incubated overnight at 37°C in L-broth containing 10µg/ml ampicillin. Eighteen hours later it was diluted for log phase growth and was induced with 10mM IPTG for 4hr. The E.coli preparation was then harvested at 5000xG for 10min, and the pellets were resuspended in 40ml of 10mM Tris-HCl, pH8.0, containing 2mM EDTA. Lysozyme was then added to a

final concentration of 0.25mg/ml, and the mixture was rotated for 30min at room temperature. The solution was made up to 0.2% of Triton X-100 with continuous mixing for an additional 10min at room temperature. An equal volume of 10mM Tris-HCl with 2mM EDTA, 50mM NaCl, and 10mM $MgCO_2$ was added with a final concentration of 2mg/ml DNAse. This was allowed to rotate for 15min at room temperature and then was spun at 1500xG for 5min. The pellet was discarded and the supernatant was spun for 30min at 10,000xG. This final pellet was then fractionated on a Sephacryl S-300 column in tandem with a Sephacryl S-400 column, after dispersion of the pellet in 0.1 M phosphate buffer, pH 6.0, with 2% SDS and 10mM dithiothreitol (DTT). The fractions were eluted at 50ml/hr, and 6-min fractions were collected for assay by analytical SDS-PAGE and immunoblotting. SDS was finally removed on a hydroxyapatite column after dilution with 0.5M phosphate buffer, pH6.8, and 1mM DTT. The purity of fractions was confirmed by SDS-PAGE and immunoblotting as above.

Immunization of mice.

Groups of six BALB/c female mice were immunized with 10µg of purified fused polypeptide in complete Freund's adjuvant (CFA). Three weeks later they were boosted with the same dose in CFA. Six weeks after the initial immunization, mice were bled and the sera were isolated. These sera were assayed at a dilution of 1/1000 and were probed against PAGE-separated placental mitochondria as above except that affinity-purified ^{125}I-goat anti-mouse Ig was used. The sera were also studied at 1/100 by immunofluorescence, using sections of HEp-2 cells and kidney tissue sections as described[1,2,5].

Nucleotide and amino acid sequence.

The cDNA insert of pRMIT was subcloned into M13,

and the nucleotide sequence as determined [16,17]. The correct frame and orientation of the insert was determined by double-stranded sequencing of an expressing clone[17]. The sequence was determined in both orientations, and use was made of synthetic oligonucleotides to prime reactions[18].

ELISA

Briefly, the purified recombinant fused polypeptide at 2µg/ml, diluted in carbonate buffer, was absorbed to Immulon 1 microtitre plates (Dynatech Laboratories, Alexandria, VA) overnight at 4°C. After blocking the non-specific sites with foetal calf serum (FCS) buffer (5% FCS, 1% BSA, 0.3% gelatin in PBS), PBC sera diluted in FCS buffer, were incubated for one hour. The plates were washed three times with PBS/O.1% tween and then incubated with each of the following mouse monoclonal antibodies specific against human heavy chain isotypes: SG-11 for IgG1, GOM-1 for IgG2, SJ-33 for IgG3, SK-44 for IgG4, MB-11 for IgM and GA-1 for IgA (Miles Scientific, Naperville, IL). The binding of mouse MoAbs were detected with peroxidase conjugated goat anti-mouse IgG (Tago, Bulingame, CA) for all except SJ-33 which was detected with peroxidase conjugated goat anti-mouse IgM (Tago, Burlingame, CA). ABTS was used as the colour substrate for the peroxidase. For detection of all isotypes of AMA, HRP-G Hulg was used in the place of isotype specific monoclonals.

Human myeloma proteins were used to obtain the optimal dilutions of the isotype specific MoAbs. Predetermined dilutions of myeloma proteins were coated onto microtitre plates and ELISA performed as before with serial dilutions of isotype specific MoAbs followed by the peroxidase conjugated reagents. The dilutions of isotype specific MoAbs which gave similar O.D. units at

approximately equal serum isotype concentrations were used in the ELISA.

To obtain the optimal serum dilutions for screening, previously screened (by immunofluorescence) AMA-positive PBC, progressive sclerosing cholangitis and normal sera were titrated by the ELISA. It was found that a serum dilution of 1:1000 yielded the highest signal to noise ratio, and this dilution was used to obtain all results. The cut-off point for negatives were determined as 2 standard deviation above the mean O.D. of normal sera.

B. RESULTS

Arrays of pRMIT in BTA224.

Subclones of pRMIT in JM101 were very immunoreactive when probed with sera from patients with PBC, whereas control clones were nonreactive. In contrast, sera from normal volunteers reacted with neither pRMIT in JM101 nor control clones. Positive colonies from arrays were used in all subsequent studies.

Specificity of pRMIT fused polypeptide.

Sera at dilutions of 1/1000 from 25 of 25 patients with PBC reacted with a 160kd fused polypeptide made in pRMIT (Table I and Fig.1). This band also reacted with a rabbit antiserum to β-galactosidase (data not shown). A number of bands corresponding to components of lower m.w. also were recognised, including one at approximately 36kd, which was apparently a breakdown product of the 160kd molecule. These lower m.w. materials were only associated with pRMIT and were immunoreactive with PBC sera. The titre of reactivity for these 25 sera ranged from 1:1000 to 1:1,000,000. With the use of the same 25 sera, the fused polypeptide was not detected in lysates of bacteria produced by non-recombinant pBTA224 or bacteria transformed with an irrelevant insert and induced

to express an abundant fused polypeptide. None of the sera from patients with systemic lupus erythematosus, rheumatoid arthritis, or chronic active hepatitis reacted with the fused protein at dilutions of 1/100, even with autoradiographic exposures of up to 4 days.

TABLE 1. Reactivity of human sera with the pRMIT fused polypeptide.

Group[a]	No.positive[b]/Total
PBC	25/25
Normal persons	0/25
Systemic lupus erythematosus	0/21
Rheumatoid arthritis	0/18
Chronic active hepatitis	0/32

a PBC sera were studied at 1/1000 sera dilution; other groups were studied at 1/100 sera dilution.

b A positive blot was one in which reactivity to a band of 160kd was readily visible after an autoradiographic exposure level of 12hr. See Figure 1.

Identification of fused polypeptide.

After absorption with the lysate of pRMIT, sera from all seven patients with PBC were shown to be depleted of antibodies reactive with the 70kd antigen (Table II). In contrast, such absorption did not change the reactivity to the 45kd or 39kd antigen. No such depletion was seen when PBC sera were absorbed against a lysate of a control clone bound to a solid support. The finding that the reaction of PBC sera with the pRMIT fused polypeptide appeared to remove detectable anti-70kd reactivity indicates that the cDNA encodes all determinants recognised by the autoantibodies to the 70kd antigen (Table II; Fig.2).

Affinity-purified antibodies.

The eluted antibodies of five different PBC sera reacted with the 70kd polypeptide of fractionated placental mitochondria and with the 160kd fused

polypeptide in pRMIT (Fig.3), further indicating that pRMIT encodes the 70kd antigen. The eluted antibodies did not react with a lysate of bacterial proteins from a clone expressing a control liver cDNA. The eluted antibodies also gave a characteristic pattern of anti-mitochondrial staining by immunofluorescence with either HEp-2 cells or kidney tissue sections.

Immune response of mice.

BALB/c mice, after injection of the pRMIT fused polypeptide, gave an antibody response to the 70kd placental mitochondrial protein. Control nonimmunized mouse sera was nonreactive (Fig.4). In addition, these sera produced a typical pattern of anti-mitochondrial immunofluorescence on both HEp-2 cells and kidney tissue sections (Fig.5).

Nucleotide and amino acid sequence.

The insert is 1370 base pairs long and consists entirely of coding region (Fig.6). The 456 amino acids would code for a polypeptide of approximately 48kd, consistent with the observed size of the fused polypeptide produced by the clone; it is thus not a full-length sequence of the antigen. The sequence contains 11% proline, and the proline is frequently found preceded by short stretches of hydrophobic amino acids such as alanine and valine, e.g., from nucleotides 54 to 102. Comparison of the sequence of the 70kd mitochondrial autoantigen with known protein and DNA sequences did not reveal any closely homologous sequences. The sequence is not present in mitochondrial DNA (data not shown), and the 70kd protein is therefore coded for by nuclear genes.

The sensitivity of ELISA was compared with immunofluorescence for 37 patients with PBC (Figure 7). ELISA was found to be approximately 250 fold more sensitive; the average titre detected by ELISA was $10^{5.4}$ whereas by immunofluorescence it was only 10^{3}.

TABLE II. Absorption of PBC sera with the pRMIT fused polypeptide

Patient	Preabsorption Titre[a]			Postabsorption Titre[a]		
	70kd	45kd	39kd	70kd	45kd	39kd
1	1:2,000	1:2,000	0	0	1:2,000	0
2	>1:20,000	1:20,000	0	1:200	1:2,000	0
3	>1:20,000	>1:20,000	1:2,000	1:200	>1:20,000	1:2,000
4	1:2,000	1:200	0	0	1:200	0
5	>1:20,000	>1:20,000	1:2,000	1:200	>1:20,000	1:2,000
6	1:2,000	0	0	0	0	0
7	>1:20,000	1:2,000	0	0	1:2,000	0

a Reactivity on immunoblots using placental mitochondria as described; absorption with control lysates does not influence the titre.

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CLAIMS:

1. A DNA molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the base sequence coding for the 70kd mitochondrial autoantigen of primary biliary cirrhosis (PBC), or an antigenically active fragment thereof.

2. A DNA molecule according to claim 1, characterised by at least a portion thereof comprising a base sequence substantially as shown in Figure 6 or Figure 8, or a fragment thereof.

3. A DNA molecule according to claim 2, characterised by at least a portion thereof comprising a base sequence corresponding substantially to nucleotides 76-679 as shown in Figure 6.

4. A DNA molecule according to claim 2, characterised by at least a portion thereof comprising a base sequence coding for the amino acid sequence:

A E I E T D K A T I G F E V Q E E G Y L.

5. A recombinant DNA molecule comprising a nucleotide sequence as claimed in any of claims 1 to 4, operatively linked to an expression control sequence.

6. An expression vector containing a nucleotide sequence as claimed in any of claims 1 to 4, operatively linked to an expression control sequence.

7. A transformed host cell containing a nucleotide sequence as claimed in any of claims 1 to 4, operatively linked to an expression control sequence.

8. A synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of primary biliary cirrhosis, or an antigenically active fragment thereof.

9. A synthetic peptide or polypeptide according to claim 8,characterised by at least a portion thereof comprising an amino acid sequence substantially as shown in Figure 6 or Figure 8, or an antigenically active fragment thereof.

10. A synthetic peptide or polypeptide according to claim 9, characterised by at least a portion thereof, comprising an amino acid sequence substantially as coded for by nucleotides 76-679 as shown in Figure 6.

11. A synthetic peptide or polypeptide according to claim 9, characterised by at least a portion thereof comprising the amino acid sequence:

A E I E T D K A T I G F E V Q E E G Y L.

12. A process for preparing a synthetic peptide or polypeptide as claimed in claim 8, which comprises cultivating a transformed host cell as claimed in claim 5 under suitable conditions, and isolating and purifying the expressed peptide or polypeptide.

13. Use of a synthetic peptide or polypeptide as claimed in any of claims 8 to 11, as an antigen in a diagnostic test.

14. Use of a synthetic peptide or polypeptide as claimed in any of claims 8 to 11, in the treatment of patients.

15. A diagnostic test for detection of anti-mitochondrial antibody in a serum sample, which comprises the steps of:

(i) contacting said serum sample with a synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of PBC, or an antigenically active fragment thereof, said synthetic peptide or polypeptide being immobilized on a support, and

(ii) detecting the presence of anti-mitochondrial antibody in said serum bound to said synthetic peptide or polypeptide.

16. A diagnostic test kit for detection of anti-mitochondrial antibody in a serum sample, which comprises:

(i) a support having immobilised thereon a synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of PBC, or an antigenically active fragment thereof, and

(ii) means for detecting the presence of anti-mitochondrial antibody in said serum bound to said synthetic peptide or polypeptide.

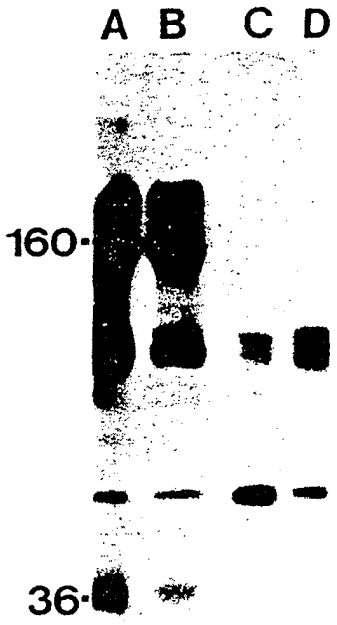

FIG. 1.

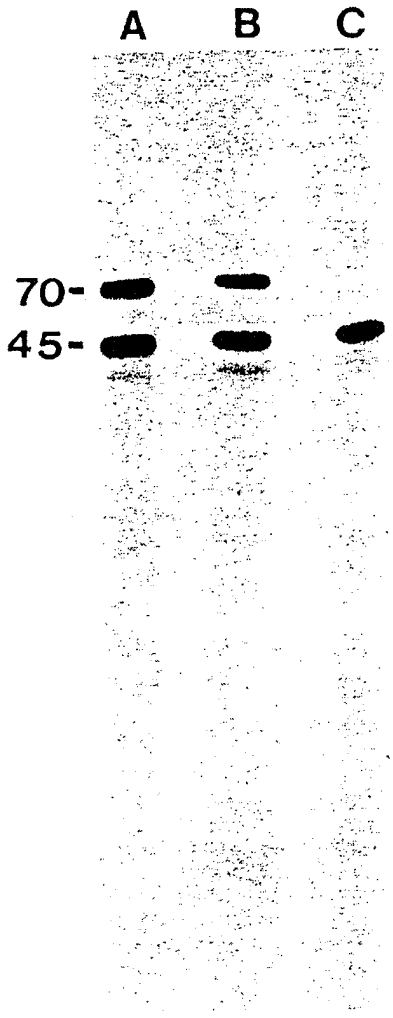

FIG. 2.

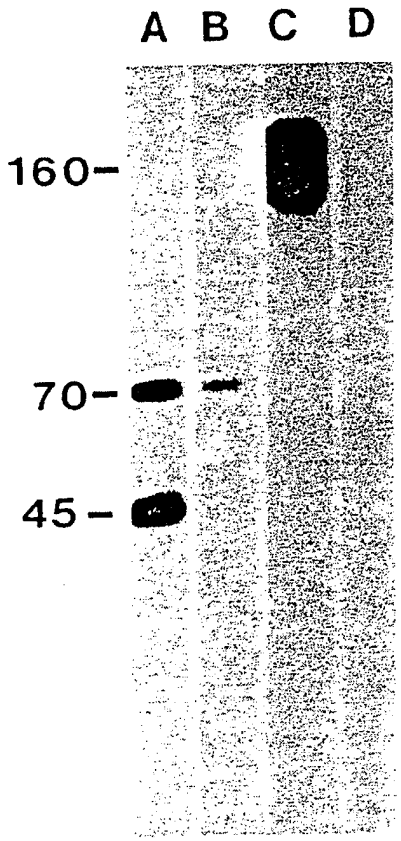

FIG. 3.

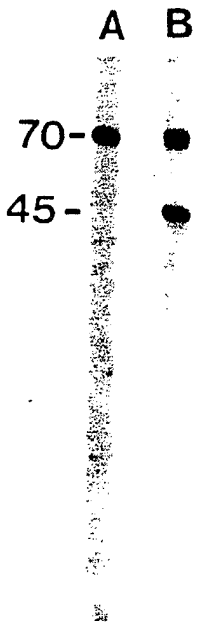

FIG. 4.

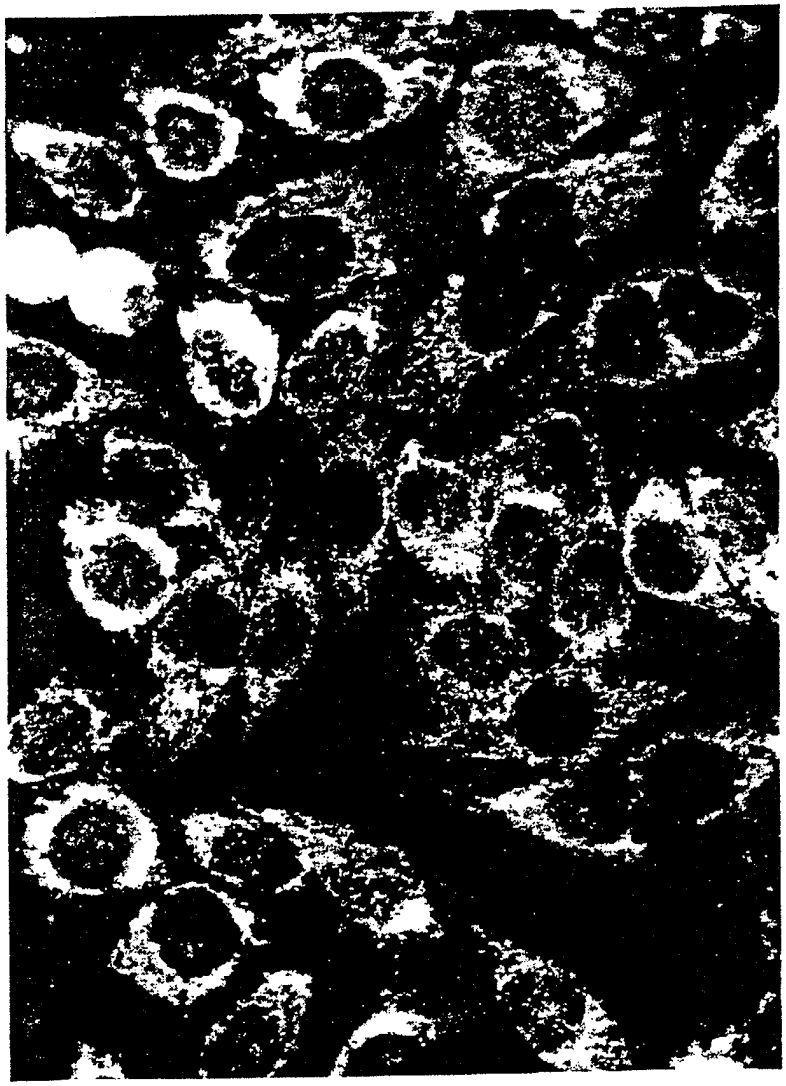

FIG. 5.

```
GlyProGluAlaPheLysAsnTyrThrLeuAspSerAlaThr
GGGCCTGAGGCTTTTAAAAATTATACATTGGATTCAGCAACA
          10        20        30        40

ProAlaAlaProSerAlaSerAlaProGlySerSerTyrPro
CCAGCTGCACCTTCTGCAAGTGCTCCAGGTAGCTCCTATCCC
         100       110       120       130

GlyThrValGlnArgTrpGluLysLysValGlyGluLysLeu
GGCACCGTCCAGAGGTGGGAAAAGAAAGTGGGAGAGAAGCTG
         190       200       210       220

GlyPheGluValGlnGluGluGlyTyrLeuAlaLysIleLeu
GGCTTTGAAGTACAAGAAGAAGGTTATCTGGCAAAAATCCTG
         280       290       300       310

IleValGluLysGlnGluAspIleAlaAlaPheAlaAspTyr
ATAGTAGAAAAACAGGAAGATATAGCAGCATTTGCAGACTAC
         370       380       390       400

ProProProValAlaAlaValProProIleProGlnProLeu
CCACCCCCAGTGGCAGCTGTTCCTCCCATCCCCCAGCCTTTA
         460       470       480       490

ValSerProLeuAlaLysLysLeuAlaAlaGluLysGlyIle
GTTAGCCCTCTTGCAAAGAAATTGGCAGCAGAGAAAGGGATT
         550       560       570       580

LysAspIleAspSerPheValProThrLysAlaAlaProAla
AAGGACATTGACTCTTTTGTGCCTACTAAGGCTGCTCCTGCC
         640       650       660       670
```

FIG. 6a.

```
AlaAlaThrGlnAlaAlaProAlaProAlaAlaAlaProAlaAlaAla
GCTGCCACACAGGCAGCCCCAGCCCCAGCTGCAGCTCCAGCTGCTGCC
      50        60        70        80        90

ValHisMetGlnIleValLeuProAlaLeuSerProThrMetThrMet
GTTCACATGCAGATTGTTCTTCCTGCCCTCTCCCCAACCATGACCATG
     140       150       160       170       180

SerGluGlyAspLeuLeuAlaGluIleGluThrAspLysAlaThrIle
AGTGAAGGAGACTTGCTGGCAGAGATAGAGACCGACAAGGCCACCATA
     230       240       250       260       270

ValProGluGlyThrArgAspValProLeuGlyThrProLeuCysIle
GTCCCTGAAGGCACAAGGGATGTTCCTCTGGGAACCCCGCTATGTATC
     320       330       340       350       360

ArgProThrGluValThrSerLeuLysProGlnAlaProProProVal
AGGCCAACAGAAGTGACCAGCTTAAAGCCACAGGCACCACCACCTGTC
     410       420       430       440       450

AlaProThrProSerAlaAlaProAlaGlyProLysGlyArgValPhe
GCACCTACCCCCTCAGCCGCTCCTGCTGGACCAAAGGGAAGGGTGTTC
     500       510       520       530       540

AspLeuThrGlnValLysGlyThrGlyProGluGlyArgIleIleLys
GACCTCACCCAAGTTAAAGGGACGGGACCAGAAGGCAGAATCATCAAG
     590       600       610       620       630

AlaAlaAlaAlaAlaProProGlyProArgValAlaProThrProAla
GCTGCAGCAGCTGCTCCCCCGGGTCCAAGAGTGGCACCAACTCCTGCA
     680       690       700       710       720
```

FIG. 6b.

```
GlyValPheIleAspIleProIleSerAsnIleArgArgVal
GGTGTCTTCATAGACATCCCCATCAGCAACATTCGTCGAGTG
        730         740         750         760

LeuSerValAspValAsnMetGlyGluValLeuLeuValArg
CTTTCTGTTGATGTAAATATGGGAGAGGTGCTGTTGGTGCGG
        820         830         840         850

AspPheIleIleLysAlaSerAlaLeuAlaCysLeuLysVal
GACTTCATCATAAAAGCTTCAGCTTTGGCCTGCCTGAAAGTT
        910         920         930         940

ValValAspValSerValAlaValSerThrProAlaGlyLeu
GTGGTTGATGTCAGCGTTGCTGTCAGTACCCCTGCAGGACTT
        1000        1010        1020        1030

AlaSerAspValValSerLeuAlaSerLysAlaArgGluGly
GCTAGTGATGTTGTTTCTTTAGCCTCCAAAGCAAGAGAGGGT
        1090        1100        1110        1120

LeuGlyMetPheGlyIleLysAsnPheSerAlaIleIleAsn
TTAGGGATGTTCGGAATTAAGAATTTCTCTGCGATTATTAAC
        1180        1190        1200        1210

IleProAlaAspAsnGluLysGlyPheAspValAlaSerVal
ATCCCTGCAGATAATGAGAAAGGCTTTGACGTGGCTAGTGTG
        1270        1280        1290        1300

LeuGluProSerGlyLeuLeu
TTGGAGCCCAGTGGCTTGCT
        1360        1370
```

FIG. 6c.

```
IleAlaGlnArgLeuMetGlnSerLysGlnThrIleProHisTyrTyr
ATTGCGCAGAGGCTCATGCAGTCGAAGCAGACTATACCTCATTATTAC
      770       780       790       800       810

LysGluLeuAsnLysMetLeuGluGlyLysGlyLysIleSerValAsn
AAGGAACTTAATAAGATGCTTGAAGGTAAAGGAAAAATCTCCGTCAAT
      860       870       880       890       900

ProGluAlaAsnSerSerTrpMetAspThrValIleArgGlnAsnHis
CCTGAAGCAAACTCATCTTGGATGGACACAGTTATACGACAAAATCAT
      950       960       970       980       990

IleThrProIleValPheAsnAlaHisIleLysGlyLeuGluThrIle
ATCACCCCTATTGTGTTTAATGCACACATAAAAGGACTGGAAACCATT
     1040      1050      1060      1070      1080

LysLeuGlnProHisGluPheGlnGlyGlyThrPheThrIleSerAsn
AAACTTCAGCCTCACGAGTTCCAGGGTGGGACATTTACAATCTCCAAC
     1130      1140      1150      1160      1170

ProProGlnAlaCysIleLeuAlaIleGlyAlaSerGluAspLysLeu
CCACCTCAGGCATGTATTTTGGCAATTGGTGCTTCCGAGGATAAACTG
     1220      1230      1240      1250      1260

MetSerValThrHisSerAlaValIleIleGluLeuTrpMetGluGln
ATGTCTGTCACACACTCAGCTGTGATCATCGAGTTGTGGATGGAGCAG
     1310      1320      1330      1340      1350
```

FIG. 6d.

SUBSTITUTE SHEET

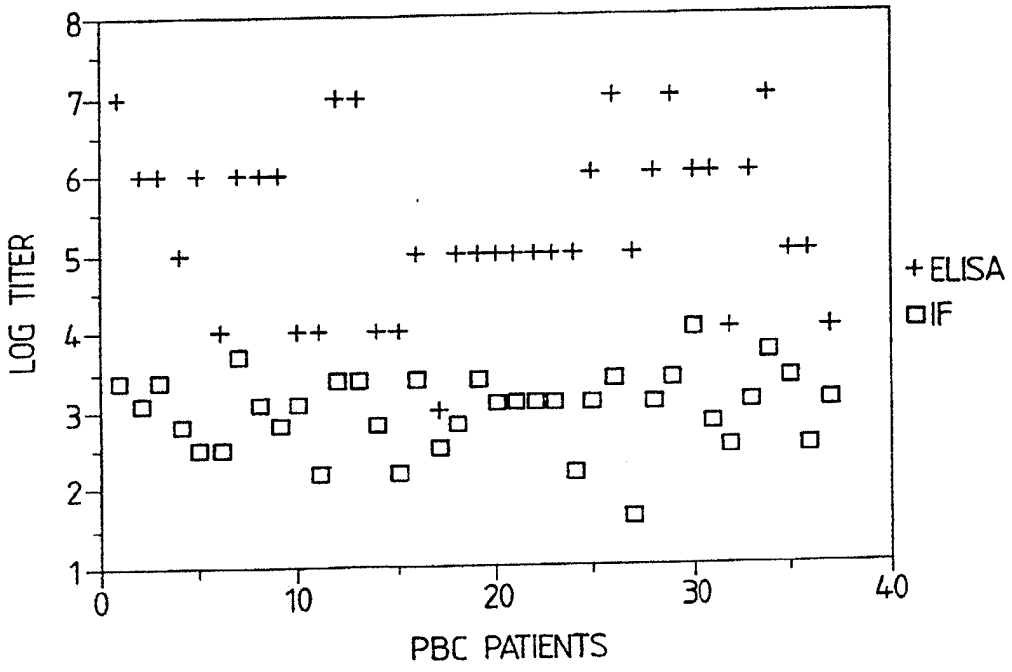

FIG. 7.

```
ProGlySerSerTyrProProHisMetGlnValLeuLeuProAla
CCTGGTAGCTCATATCCCCCTCACATGCAGGTACTTCTTCCTGCC
        10        20        30        40

LysValGlyGluLysLeuSerGluGlyAspLeuLeuAlaGluIle
AAAGTGGGTGAGAAGCTAAGTGAAGGAGACTTACTGGCAGAGATA
       100       110       120       130

TyrLeuAlaLysIleLeuValProGluGlyThrArgAspValPro
TATCTGGCAAAAATCCTGGTCCCTGAAGGCACAAGAGATGTCCCT
       190       200       210       220

SerAlaPheAlaAspTyrArgProThrGluValThrAspLeuLys
TCAGCATTTGCTGACTATAGGCCAACCGAAGTAACAGATTTAAAA
       280       290       300       310

ProThrProGlnProLeuAlaProThrProSerAlaProCysPro
CCAACTCCCCAGCCTTTAGCTCCTACACCTTCAGCACCCTGCCCA
       370       380       390       400

AlaLysLysLeuAlaValGluLysGlyIleAspLeuThrGlnVal
GCAAAGAAGTTGGCAGTAGAGAAAGGGATTGATCTTACACAAGTA
       460       470       480       490

SerPheValProSerLysValAlaProAlaProAlaAlaValVal
TCTTTTGTGCCTAGTAAAGTTGCTCCTGCTCCGGCAGCTGTTGTG
       550       560       570       580

ThrAspIleProIleSerAsnIleArgArgValIleAlaGlnArg
ACAGATATCCCAATCAGCAACATTCGTCGGGTTATTGCACAGCGA
       640       650       660       670

AspValAsnMetGlyGluValLeuLeuValArgLysGluLeuAsn
GATGTAAATATGGGAGAAGTTTTGTTGGTACGGAAAGAACTTAAT
       730       740       750       760

IleLysAlaSerAlaLeuAlaCysLeuLysValProGluAlaAsn
ATAAAAGCTTCAGCTTTGGCATGTTTAAAAGTTCCCGAAGCAAAT
       820       830       840       850

ValSerValAlaValSerThrProAlaGlyLeuIleThrProIle
GTCAGTGTTGCGGTCAGTACTCCTGCAGGACTCATCACACCTATT
       910       920       930       940
```

FIG. 8a.

11/11

```
LeuSerProThrMetThrMetGlyThrValGlnArgTrpGluLys
CTCTCTCCCACCATGACCATGGGCACAGTTCAGAGATGGGAAAAA
   50        60        70        80        90

GluThrAspLysAlaThrIleGlyPheGluValGlnGluGluGly
GAAACTGACAAAGCCACTATAGGTTTTGAAGTACAGGAAGAAGGT
  140       150       160       170       180

LeuGlyThrProLeuCysIleIleValGluLysGluAlaAspIle
CTAGGAACCCCACTCTGTATCATTGTAGAAAAAGAGGCAGATATA
  230       240       250       260       270

ProGlnValProProProThrProProProValAlaAlaValPro
CCACAAGTGCCACCACCTACCCCACCCCCGGTGGCCGCTGTTCCT
  320       330       340       350       360

AlaThrProAlaGlyProLysGlyArgValPheValSerProLeu
GCTACTCCTGCTGGACCAAAGGGAAGGGTGTTTGTTAGCCCTCTT
  410       420       430       440       450

LysGlyThrGlyProAspGlyArgIleThrLysLysAspIleAsp
AAAGGGACAGGACCAGATGGTAGAATCACCAAGAAGGATATCGAC
  500       510       520       530       540

ProProThrGlyProGlyMetAlaProValProThrGlyValPhe
CCTCCCACAGGTCCTGGAATGGCACCAGTTCCTACAGGTGTCTTC
  590       600       610       620       630

LeuMetGlnSerLysGlnThrIleProHisTyrTyrLeuSerIle
TTAATGCAATCAAAGCAAACCATACCTCATTATTACCTTTCTATC
  680       690       700       710       720

LysIleLeuGluGlyArgSerLysIleSerValAsnAspPheIle
AAGATATTAGAAGGGAGAAGCAAAATTTCTGTCAATGACTTCATC
  770       780       790       800       810

SerSerTrpMetAspThrValIleArgGlnAsnHisValValAsp
TCTTCTTGGATGGACACAGTTATAAGACAAAATCATGTTGTTGAT
  860       870       880       890       900

ValPheAsnAlaHisIleLys
GTGTTTAATGCACATATAAAA
  950       960
```

FIG. 8b.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00427

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 15/00, C07G 17/00, C07H 21/04, C07K 15/12, 13/00, 7/08, C12N 9/10, 1/20, G01N 33/543

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA : Keyword: Biliary Cirrhosis (Derwent Databases)

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

AU: IPC as above; Chemical Abstracts : Keyword: Biliary Cirrhosis; GenBank, NBRF, EMBL, Kyoto Databases

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Chemical Abstracts, Volume 107, no. 3, issued 20 July 1987 (Columbus, Ohio, U.S.A.), E.M. Gershwin et al, "Identification and specificity of a cDNA encoding the 70kD mitochondrial antigen recognized in primary biliary cirrhosis", see page 180, column 1, abstract no. 18762n, J. Immunol., 1987, 138(10), 3525-31 (Eng.).	(1-12)
X	Mendel-Hartvig, I. et al. "Primary biliary cirrhosis: further biochemical and immunological characterization of mitochondrial antigens", Clinical and Experimental Immunology, Volume 62, issued 1985 (Blackwell Scientific Publications, Oxford, England), see pages 371 to 379.	(8)
X	Frazer, I.H. et al. "Reactivity of Anti-mitochondrial Autoantibodies in Primary Biliary Cirrhosis : Definition of Two Novel Mitochondrial Polypeptide Antigens", Journal of Immunology, Volume 135, No. 3, issued 1985 September (Williams & Wilkins Co., Baltimore, U.S.A.), see pages 1739 to 1745.	(8)

(continued)

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6 April 1988 (06.04.88)	(11.04.88) 11 APRIL 1988
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	S.D. BARKER

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A — Alderuccio, F. et al. "Identification and Characterization of Mitochondria Autoantigens in Progressive Systemic Sclerosis : Identity with the 72,000 Dalton Autoantigen in Primary Biliary Cirrhosis", Journal of Immunology, Volume 137, no. 6, issued 1986 September, (Williams & Wilkins Co., Baltimore, U.S.A.), see pages 1855 to 1859.

A — Baum, H. et al. "The PBC-Specific Antigen", Molecular Aspects of Medicine, Volume 8, no. 3, issued 1985 (Pergamon Press, Ltd, Oxford, England), see pages 201 to 234.

(continued)

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 13 to 16, because they relate to subject matter not required to be searched by this Authority, namely:

the matter claimed is excluded subject matter, that is, diagnostic methods etc. under Rule 39 of the P.C.T.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Berg, P.A. et al. "ATPase-Associated Antigen (M2): Marker Antigen for Serological Diagnosis of Primary Biliary Cirrhosis", The Lancet, Volume 2 (8313), issued 1982 December, (Lancet Ltd, London, England), see pages 1423 to 1426.	
A	Sayers, T. et al. "Antimitochondrial Antibodies (AMA) in Primary Biliary Cirrhosis. I. Separation of the PBC Antigen Activity from mitochondrial ATPase Activity", Journal of Bioenergetics and Biomembranes, Volume 13, nos. 5/6, issued 1981, (Plenum Publishing Corp., New York, U.S.A.), see pages 255 to 267.	

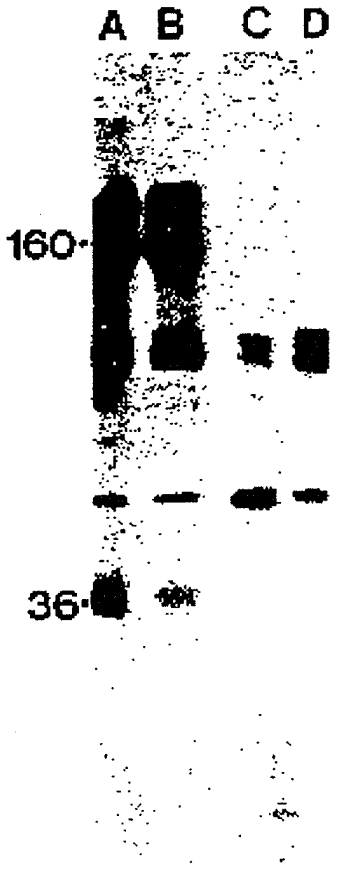

FIG. 1.

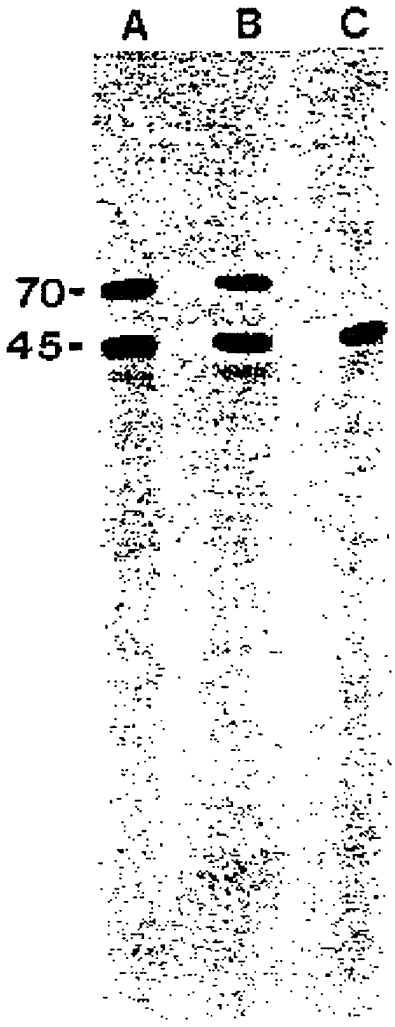

FIG. 2.

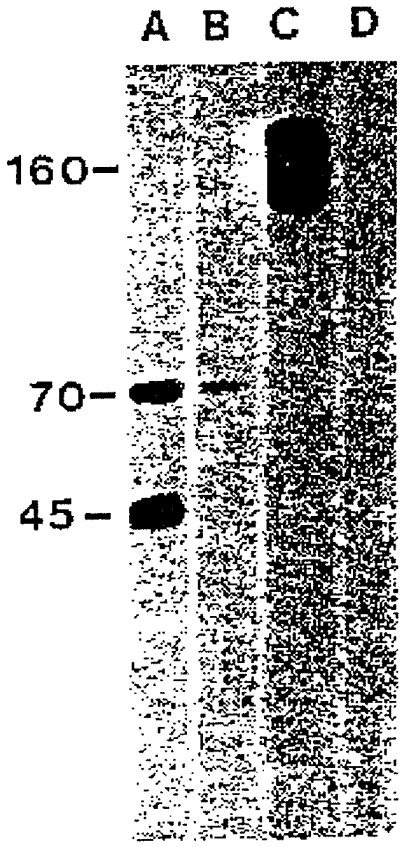

FIG. 3.

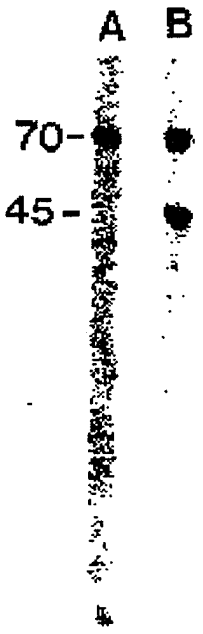

FIG. 4.

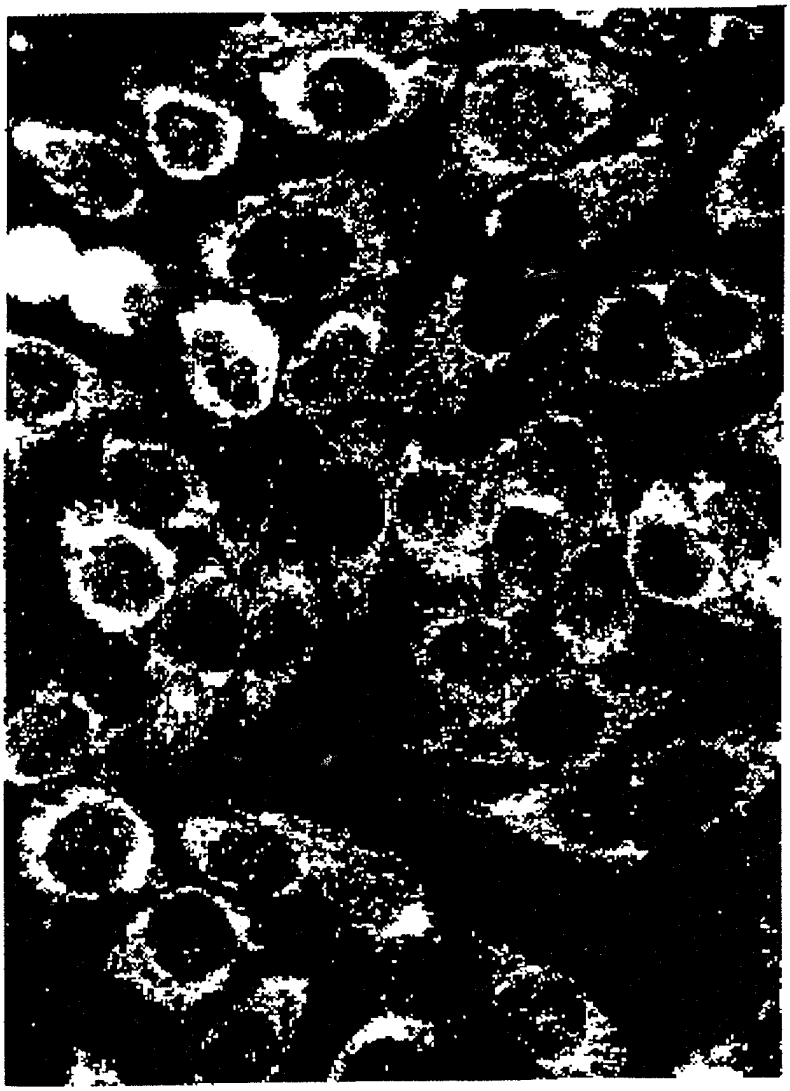

FIG. 5.

```
GlyProGluAlaPheLysAsnTyrThrLeuAspSerAlaThr
GGGCCTGAGGCTTTTAAAAATTATACATTGGATTCAGCAACA
          10        20        30        40

ProAlaAlaProSerAlaSerAlaProGlySerSerTyrPro
CCAGCTGCACCTTCTGCAAGTGCTCCAGGTAGCTCCTATCCC
         100       110       120       130

GlyThrValGlnArgTrpGluLysLysValGlyGluLysLeu
GGCACCGTCCAGAGGTGGGAAAAGAAAGTGGGAGAGAAGCTG
         190       200       210       220

GlyPheGluValGlnGluGluGlyTyrLeuAlaLysIleLeu
GGCTTTGAAGTACAAGAAGAAGGTTATCTGGCAAAAATCCTG
         280       290       300       310

IleValGluLysGlnGluAspIleAlaAlaPheAlaAspTyr
ATAGTAGAAAAACAGGAAGATATAGCAGCATTTGCAGACTAC
         370       380       390       400

ProProProValAlaAlaValProProIleProGlnProLeu
CCACCCCCAGTGGCAGCTGTTCCTCCCATCCCCCAGCCTTTA
         460       470       480       490

ValSerProLeuAlaLysLysLeuAlaAlaGluLysGlyIle
GTTAGCCCTCTTGCAAAGAAATTGGCAGCAGAGAAAGGGATT
         550       560       570       580

LysAspIleAspSerPheValProThrLysAlaAlaProAla
AAGGACATTGACTCTTTTGTGCCTACTAAGGCTGCTCCTGCC
         640       650       660       670
```

FIG. 6a.

```
AlaAlaThrGlnAlaAlaProAlaProAlaAlaAlaProAlaAlaAla
GCTGCCACACAGGCAGCCCCAGCCCCAGCTGCAGCTCCAGCTGCTGCC
      50        60        70        80        90

ValHisMetGlnIleValLeuProAlaLeuSerProThrMetThrMet
GTTCACATGCAGATTGTTCTTCCTGCCCTCTCCCCAACCATGACCATG
     140       150       160       170       180

SerGluGlyAspLeuLeuAlaGluIleGluThrAspLysAlaThrIle
AGTGAAGGAGACTTGCTGGCAGAGATAGAGACCGACAAGGCCACCATA
     230       240       250       260       270

ValProGluGlyThrArgAspValProLeuGlyThrProLeuCysIle
GTCCCTGAAGGCACAAGGGATGTTCCTCTGGGAACCCCGCTATGTATC
     320       330       340       350       360

ArgProThrGluValThrSerLeuLysProGlnAlaProProProVal
AGGCCAACAGAAGTGACCAGCTTAAAGCCACAGGCACCACCACCTGTC
     410       420       430       440       450

AlaProThrProSerAlaAlaProAlaGlyProLysGlyArgValPhe
GCACCTACCCCCTCAGCCGCTCCTGCTGGACCAAAGGGAAGGGTGTTC
     500       510       520       530       540

AspLeuThrGlnValLysGlyThrGlyProGluGlyArgIleIleLys
GACCTCACCCAAGTTAAAGGGACGGGACCAGAAGGCAGAATCATCAAG
     590       600       610       620       630

AlaAlaAlaAlaAlaProProGlyProArgValAlaProThrProAla
GCTGCAGCAGCTGCTCCCCCGGGTCCAAGAGTGGCACCAACTCCTGCA
     680       690       700       710       720
```

FIG. 6b.

7/11

```
GlyValPheIleAspIleProIleSerAsnIleArgArgVal
GGTGTCTTCATAGACATCCCCATCAGCAACATTCGTCGAGTG
      730       740       750       760

LeuSerValAspValAsnMetGlyGluValLeuLeuValArg
CTTTCTGTTGATGTAAATATGGGAGAGGTGCTGTTGGTGCGG
      820       830       840       850

AspPheIleIleLysAlaSerAlaLeuAlaCysLeuLysVal
GACTTCATCATAAAAGCTTCAGCTTTGGCCTGCCTGAAAGTT
      910       920       930       940

ValValAspValSerValAlaValSerThrProAlaGlyLeu
GTGGTTGATGTCAGCGTTGCTGTCAGTACCCCTGCAGGACTT
     1000      1010      1020      1030

AlaSerAspValValSerLeuAlaSerLysAlaArgGluGly
GCTAGTGATGTTGTTTCTTTAGCCTCCAAAGCAAGAGAGGGT
     1090      1100      1110      1120

LeuGlyMetPheGlyIleLysAsnPheSerAlaIleIleAsn
TTAGGGATGTTCGGAATTAAGAATTTCTCTGCGATTATTAAC
     1180      1190      1200      1210

IleProAlaAspAsnGluLysGlyPheAspValAlaSerVal
ATCCCTGCAGATAATGAGAAAGGCTTTGACGTGGCTAGTGTG
     1270      1280      1290      1300

LeuGluProSerGlyLeuLeu
TTGGAGCCCAGTGGCTTGCT
     1360      1370
```

FIG. 6c.

SUBSTITUTE SHEET

8/11

```
IleAlaGlnArgLeuMetGlnSerLysGlnThrIleProHisTyrTyr
ATTGCGCAGAGGCTCATGCAGTCGAAGCAGACTATACCTCATTATTAC
      770       780       790       800       810

LysGluLeuAsnLysMetLeuGluGlyLysGlyLysIleSerValAsn
AAGGAACTTAATAAGATGCTTGAAGGTAAAGGAAAAATCTCCGTCAAT
      860       870       880       890       900

ProGluAlaAsnSerSerTrpMetAspThrValIleArgGlnAsnHis
CCTGAAGCAAACTCATCTTGGATGGACACAGTTATACGACAAAATCAT
      950       960       970       980       990

IleThrProIleValPheAsnAlaHisIleLysGlyLeuGluThrIle
ATCACCCCTATTGTGTTTAATGCACACATAAAAGGACTGGAAACCATT
     1040      1050      1060      1070      1080

LysLeuGlnProHisGluPheGlnGlyGlyThrPheThrIleSerAsn
AAACTTCAGCCTCACGAGTTCCAGGGTGGGACATTTACAATCTCCAAC
     1130      1140      1150      1160      1170

ProProGlnAlaCysIleLeuAlaIleGlyAlaSerGluAspLysLeu
CCACCTCAGGCATGTATTTTGGCAATTGGTGCTTCCGAGGATAAACTG
     1220      1230      1240      1250      1260

MetSerValThrHisSerAlaValIleIleGluLeuTrpMetGluGln
ATGTCTGTCACACACTCAGCTGTGATCATCGAGTTGTGGATGGAGCAG
     1310      1320      1330      1340      1350
```

FIG. 6d.

SUBSTITUTE SHEET

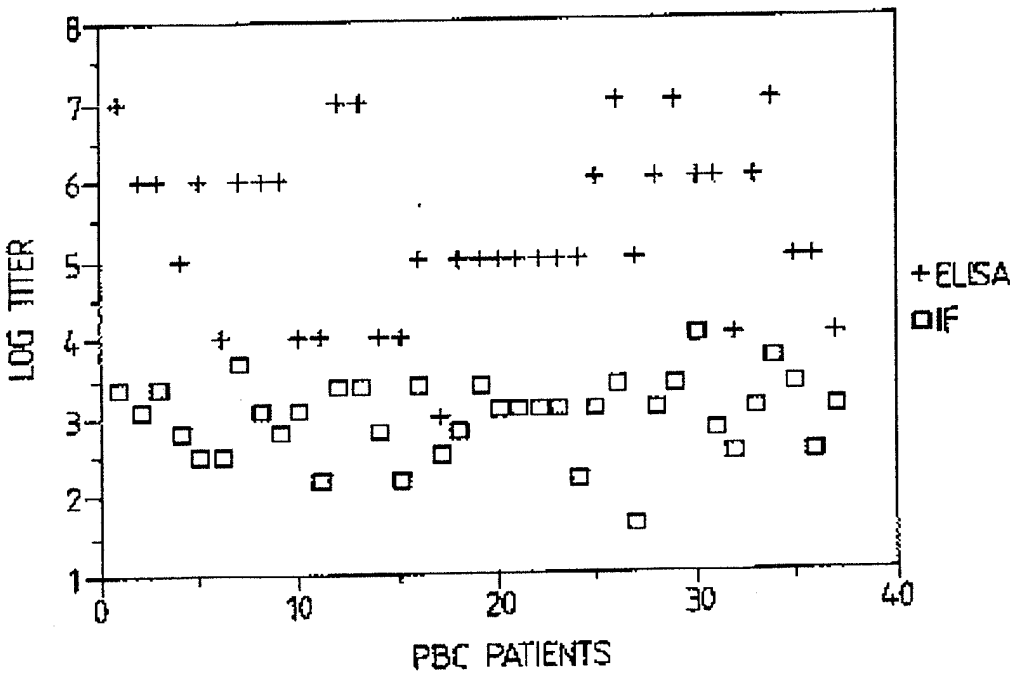

FIG. 7.

10/11

```
ProGlySerSerTyrProProHisMetGlnValLeuLeuProAla
CCTGGTAGCTCATATCCCCCTCACATGCAGGTACTTCTTCCTGCC
          10        20        30        40

LysValGlyGluLysLeuSerGluGlyAspLeuLeuAlaGluIle
AAAGTGGGTGAGAAGCTAAGTGAAGGAGACTTACTGGCAGAGATA
         100       110       120       130

TyrLeuAlaLysIleLeuValProGluGlyThrArgAspValPro
TATCTGGCAAAAATCCTGGTCCCTGAAGGCACAAGAGATGTCCCT
         190       200       210       220

SerAlaPheAlaAspTyrArgProThrGluValThrAspLeuLys
TCAGCATTTGCTGACTATAGGCCAACCGAAGTAACAGATTTAAAA
         280       290       300       310

ProThrProGlnProLeuAlaProThrProSerAlaProCysPro
CCAACTCCCCAGCCTTTAGCTCCTACACCTTCAGCACCCTGCCCA
         370       380       390       400

AlaLysLysLeuAlaValGluLysGlyIleAspLeuThrGlnVal
GCAAAGAAGTTGGCAGTAGAGAAAGGGATTGATCTTACACAAGTA
         460       470       480       490

SerPheValProSerLysValAlaProAlaProAlaAlaValVal
TCTTTTGTGCCTAGTAAAGTTGCTCCTGCTCCGGCAGCTGTTGTG
         550       560       570       580

ThrAspIleProIleSerAsnIleArgArgValIleAlaGlnArg
ACAGATATCCCAATCAGCAACATTCGTCGGGTTATTGCACAGCGA
         640       650       660       670

AspValAsnMetGlyGluValLeuLeuValArgLysGluLeuAsn
GATGTAAATATGGGAGAAGTTTTGTTGGTACGGAAAGAACTTAAT
         730       740       750       760

IleLysAlaSerAlaLeuAlaCysLeuLysValProGluAlaAsn
ATAAAAGCTTCAGCTTTGGCATGTTTAAAAGTTCCCGAAGCAAAT
         820       830       840       850

ValSerValAlaValSerThrProAlaGlyLeuIleThrProIle
GTCAGTGTTGCGGTCAGTACTCCTGCAGGACTCATCACACCTATT
         910       920       930       940
```

FIG. 8a.

```
LeuSerProThrMetThrMetGlyThrValGlnArgTrpGluLys
CTCTCTCCCACCATGACCATGGGCACAGTTCAGAGATGGGAAAAA
   50        60        70        80        90

GluThrAspLysAlaThrIleGlyPheGluValGlnGluGluGly
GAAACTGACAAAGCCACTATAGGTTTTGAAGTACAGGAAGAAGGT
  140       150       160       170       180

LeuGlyThrProLeuCysIleIleValGluLysGluAlaAspIle
CTAGGAACCCCACTCTGTATCATTGTAGAAAAAGAGGCAGATATA
  230       240       250       260       270

ProGlnValProProProThrProProProValAlaAlaValPro
CCACAAGTGCCACCACCTACCCCACCCCCGGTGGCCGCTGTTCCT
  320       330       340       350       360

AlaThrProAlaGlyProLysGlyArgValPheValSerProLeu
GCTACTCCTGCTGGACCAAAGGGAAGGGTGTTTGTTAGCCCTCTT
  410       420       430       440       450

LysGlyThrGlyProAspGlyArgIleThrLysLysAspIleAsp
AAAGGGACAGGACCAGATGGTAGAATCACCAAGAAGGATATCGAC
  500       510       520       530       540

ProProThrGlyProGlyMetAlaProValProThrGlyValPhe
CCTCCCACAGGTCCTGGAATGGCACCAGTTCCTACAGGTGTCTTC
  590       600       610       620       630

LeuMetGlnSerLysGlnThrIleProHisTyrTyrLeuSerIle
TTAATGCAATCAAAGCAAACCATACCTCATTATTACCTTTCTATC
  680       690       700       710       720

LysIleLeuGluGlyArgSerLysIleSerValAsnAspPheIle
AAGATATTAGAAGGGAGAAGCAAAATTTCTGTCAATGACTTCATC
  770       780       790       800       810

SerSerTrpMetAspThrValIleArgGlnAsnHisValValAsp
TCTTCTTGGATGGACACAGTTATAAGACAAAATCATGTTGTTGAT
  860       870       880       890       900

ValPheAsnAlaHisIleLys
GTGTTTAATGCACATATAAAA
  950       960
```

FIG. 8b.